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SYNTHESIS OF MALTO-OLIGOSACCHARIDES VIA THE ACCEPTOR REACTION CATALYZED BY CYCLODEXTRIN GLYCOSYLTRANSFERASES

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The disproportionation activity (intermolecular transglycosylation) of cyclomaltodextrin glycosyltransferases (CGTases) from *Thermoanaerobacter* sp. and *Bacillus circulans* strain 251 was studied. Using soluble starch as donor, the CGTase from *Thermoanaerobacter* sp. showed the highest transglycosylation activity with all the malto-oligosaccharides tested as acceptors. At ratios of starch:D-glucose from 2:1 to 1:2 (w/w), the formation of cyclodextrins was completely inhibited, and a homologous series of malto-oligosaccharides (G_n) was produced. The conversion of starch into acceptor products was in the range of 63–79% in 48 h. The degree of polymerisation of malto-oligosaccharides formed could be modulated by the ratio of starch:D-glucose provided; at a ratio of 1:2 (w/w), the reaction was quite selective for the formation of G2–G3.

Keywords: Cyclodextrin glycosyltransferase; *Thermoanaerobacter*; *Bacillus circulans*; Malto-oligosaccharide; Disproportionation; Acceptor

INTRODUCTION

Cyclomaltodextrin glycosyltransferase [1,4- α -D-glucan 4- α -D-(1,4- α -glucano)-transferase(cyclizing), EC 2.4.1.19, CGTase] is a unique bacterial

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enzyme that catalyses three transferase reactions (Tonkova, 1998). Cyclization is an intramolecular transglycosylation in which the CGTase converts starch into cyclodextrins (CDs). Coupling is the reaction where a CD molecule is cut open and combined with a linear oligosaccharide to produce a longer linear oligosaccharide. Disproportionation is the transfer of part of a linear oligosaccharide chain to another linear oligosaccharide acting as acceptor. In addition, CGTase displays a weak saccharifying activity (hydrolysis of starch).

CGTase is able to use various carbohydrates as acceptors, *e.g.*, xylose (Kitahata and Okada, 1976), sucrose (Okada, 1987), maltose (Vetter *et al.*, 1992) and lactose (Shibuya *et al.*, 1993), yielding oligosaccharides of strong industrial interest. In recent years compounds of a different nature (natural glucosides, sugar alcohols, vitamins, flavonoids, *etc.*), including inositol (Sato *et al.*, 1991), rutin (Suzuki and Suzuki, 1991), ascorbic acid (Aga *et al.*, 1991), stevioside (Lee *et al.*, 1993) and hesperidin (Kometani *et al.*, 1994), also have been successfully used as acceptors in CGTase catalyzed intermolecular transglycosylation reactions (coupling or disproportionation). The resulting transglycosylated saccharides and glucosides usually exhibit a better functionality than the parent acceptor molecules, *e.g.*, increased water solubility, stability or bifidogenic properties (Park *et al.*, 1998).

When D-glucose is used as acceptor and starch as donor, maltose and other linear malto-oligosaccharides (MOS, composed of α -D-glucose residues linked by $\alpha(1 \rightarrow 4)$ glycosidic bonds) are produced. MOS constitute an important class of oligosaccharides in the food industry (production in 1995 was 10,000 tonnes (Crittenden and Playne, 1996)). These compounds reduce the levels of intestinal putrefactive bacteria such as *Clostridium perfringens* and members of the family Enterobacteriaceae, improving colonic conditions in humans (Nakakuki, 1993). Traditionally, MOS are produced by the action of debranching enzymes such as pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68) combined with hydrolysis by various α -amylases (Crittenden and Playne, 1996).

Thermoanaerobacter sp. (a thermophilic anaerobic bacterium) produces a highly thermostable CGTase. This enzyme is able to degrade starch under jet-cooking industrial conditions, thus avoiding the use of α -amylase pretreatment for the liquefaction of starch (Starnes *et al.*, 1991). This allows the enzymatic synthesis of CDs using a single enzyme (Zamost *et al.*, 1991). The optimum temperature for this enzyme is 85°C at pH 5.5 (Norman and Jorgensen, 1992). The *Thermoanaerobacter* CGTase displays a high disproportionation activity (Alcalde *et al.*, 1999).

This paper describes an analysis of the disproportionation reaction of CGTase from *Thermoanaerobacter* sp. The one-step synthesis of MOS

(degree of polymerisation (d.p.) 2–10), using starch as glucosyl donor and D-glucose as acceptor, appears feasible.

MATERIALS AND METHODS

Materials

CGTase from *Thermoanaerobacter* sp. was kindly provided by Novo Nordisk (Toruzyme 3.0 L, batch ACN00019). Pure CGTase from *Bacillus circulans* (strain 251) was obtained as described by Penninga *et al.* (1996). Phenolphthalein, bromocresol green, malto-oligosaccharides (G1–G7), α -, β - and γ -CD, methyl α -D-glucopyranoside and amyloglucosidase (E.C. 3.2.1.3) from *Aspergillus niger*, were purchased from Sigma. Glucose GOD-Perid reagent, *p*-nitrophenyl- α -D-maltoheptaoside-4, 6-O-ethylidene (EPS) and α -glucosidase (E.C. 3.2.1.20) from *Saccharomyces cerevisiae*, were from Boehringer Mannheim. 3,5-Dinitrosalicylic acid (DNS) was from Fluka. Methyl orange was from Aldrich. Ampholites (pH 3–9) and pI markers for isoelectric focusing were from Bio-Rad. Potato soluble starch (Paselli SA2) was kindly donated by Avebe (Foxhol, The Netherlands).

Methods

Enzyme Purification

CGTases were purified by affinity chromatography using a column (1 × 12 cm) packed with Sepharose-6FF (Pharmacia) covalently coupled to α -cyclodextrin, with a maximal capacity of 3.5 mg protein per ml of gel. The gel was activated with α -cyclodextrin as described by Sundberg and Porath (1974). Once the crude enzyme was loaded, the column was washed with 200 ml of 10 mM sodium acetate buffer (pH 5.5) at 2.5 ml/min, and the bound CGTase was further eluted with 50 ml of the same buffer containing α -cyclodextrin (10 mg/ml). Fractions containing the CGTase were pooled and stored at –20°C (in the presence of α -CD).

The concentration of the protein was determined by the Bio-Rad assay with immunoglobulin G (IgG) as standard.

Assays of Initial Activities

The *Thermoanaerobacter* sp. CGTase activities were measured at 85°C by incubating appropriately diluted enzyme with substrate solutions in 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM CaCl₂. Reactions were

followed for 5–10 min. At time intervals, aliquots were removed and assayed using the corresponding reagent. With the CGTase from *B. circulans* the same protocol was employed at 50°C in 10 mM sodium citrate buffer (pH 6.0).

Cyclization Activity The production of cyclodextrins was detected spectrophotometrically *via* the formation of inclusion complexes with several organic compounds. Paselli SA2 (partially hydrolyzed potato starch, with an average degree of polymerisation of 50) was used as substrate at concentrations of 5% (w/v) for β - and γ -CD assays, and 2% (w/v) for α -CD. α -CD was determined at 490 nm on the basis of its ability to form a stable, colourless inclusion complex with methyl orange (Hirai *et al.*, 1981). β -CD was determined at 552 nm on the basis of its ability to form a stable, colourless inclusion complex with phenolphthalein (Penninga *et al.*, 1995). γ -CD was determined measuring the colour increase at 630 nm due to the formation of an inclusion complex with bromocresol green (Kato and Horikoshi, 1984). One unit of activity was defined as the amount of enzyme able to produce 1 μ mol of $\alpha/\beta/\gamma$ -CD per min under the corresponding conditions.

Coupling The reaction was based on the method described by Nakamura *et al.* (1993). α - or β -cyclodextrins (2.5 mM) were used as donors and methyl- α -D-glucopyranoside (10 mM) as acceptor. The linear oligo-saccharide formed was converted into single glucose units by the action of amyloglucosidase. The concentration of glucose was accurately detected with the glucose/GOD-Perid reagent. One unit of activity was defined as the amount of enzyme able to convert 1 μ mol of cyclodextrin per min under the above conditions.

Disproportionation The reaction was based on the method described by Nakamura *et al.* (1994). EPS (*p*-nitrophenyl- α -D-maltoheptaoside-4-6-O-ethylidene, 3 mM) was used as donor and maltose (10 mM) as acceptor. In this reaction EPS is cleaved and the maltose is coupled to the free reducing end. The *p*-nitrophenol may be cleaved from the reaction product by the action of α -glucosidase. One unit of activity was defined as the amount of enzyme able to release 1 μ mol of *p*-nitrophenol per min under these conditions.

Saccharifying Hydrolytic activity was assessed using 2% potato soluble starch as substrate, measuring the increase in reducing ends. The reducing power was accurately measured with dinitrosalicylic acid (Bernfeld,

1955). One unit of activity was defined as the amount of enzyme able to release 1 μ mol of reducing end per min under the corresponding conditions.

Production Assay

The CGTase from *Thermoanaerobacter* sp. (0.7 μ g/ml) was incubated at 85°C (or 60°C) with 10% (w/v) soluble starch in 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM CaCl₂. At different times aliquots were taken and mixed with the same volume of 0.4 N NaOH to quench the reaction. Samples were centrifuged during 15 min at 8.500 \times g and analyzed by HPLC. Reactions were also performed in the presence of different amounts of D-glucose.

HPLC Analysis

Samples were analyzed by high-performance liquid chromatography (HPLC) using a ternary pump (Varian) and two Aminex HPX-42A columns (300 \times 7.8 mm, Bio-rad) put in series. Water was used as mobile phase (0.7 ml/min). The column temperature was kept constant at 85°C. Detection was performed using a refractive-index detector (Varian). Integration was carried out using the Varian Star 4.0 software.

Thin-layer Chromatography

Reactions were also followed by analytical TLC performed on silica gel 60 plates (Merck) using water/ethanol/butanol (2:5:3, v/v/v) as eluent (Del Rio *et al.*, 1997). Spots were detected by immersion in a solution of orcinol/ferric chloride (Bial's reagent) diluted with ethanol (4 vol.), drying and heating at 120°C for 5 min.

RESULTS AND DISCUSSION

Purification and Characterisation of CGTases

CGTases were purified by affinity chromatography yielding pure enzymes (assessed by SDS/PAGE and isoelectrofocusing) with specific β -cyclodextrin forming activities of 275 U/mg for *Thermoanaerobacter* sp. and 255 U/mg for *B. circulans*.

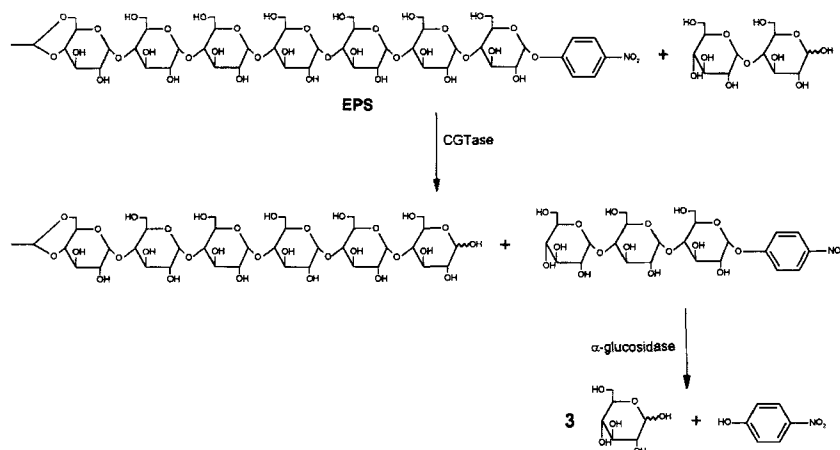
The cyclization, coupling, disproportionation and saccharifying activities of both CGTases are summarized in Table I. All the assays were followed for a short time (< 10 min) to avoid interferences between the different reactions. No reliable γ -coupling activities could be determined because a partial hydrolysis of γ -CD was catalyzed by the amyloglucosidase used, disturbing the assay. As reported previously (Alcalde *et al.*, 1999) it is noteworthy that the hydrolytic activity of CGTase from *Thermoanaerobacter* sp. (85 U/mg) is much higher than the values observed for other CGTases (1–10 U/mg). Whereas the CGTase from *B. circulans* shows a disproportionation activity (290 U/mg) in the range of other CGTases (*e.g.*, *Thermoanaerobacterium thermosulfurigenes* CGTase displays a disproportionation activity of 330 U/mg (Wind *et al.*, 1998)), the CGTase from *Thermoanaerobacter* sp. exhibits a 5-fold higher specific activity. This is the highest value ever reported for a CGTase. The main reaction catalyzed by CGTase from *Thermoanaerobacter* sp. is thus disproportionation, not cyclization. Cyclodextrins are produced only when the non-reducing end of cleaved amylose bound to the enzyme is recognized by the acceptor binding site (Nakamura *et al.*, 1994). In view of these observations, we decided to study malto-oligosaccharide synthesis by *Thermoanaerobacter* CGTase in more detail.

Effect of the Acceptor in the Disproportionation Activity

In the disproportionation reaction assay, an heptasaccharide derivative (EPS) is used as donor (Scheme 1). This substrate – blocked at the non-reducing end to avoid its action as acceptor – contains a *p*-nitrophenyl group at its reducing end to quantify the transglycosylation. This reaction can be described according to a ping-pong bi–bi mechanism (van der Veen *et al.*, 2000). The individual steps in this reaction have been deduced from a crystal structure of CGTase from *B. circulans* (Knegtel *et al.*, 1995): soaking

TABLE I Initial activities of CGTases at 85°C. Reaction conditions are described in Materials and Methods

CGTase	Activity (U/mg protein)						Saccharifying
	Cyclization			Coupling		Disproportionation	
	α -CD	β -CD	γ -CD	α -CD	β -CD		
<i>Thermoanaerobacter</i> sp.	315	275	165	295	55	1550	85
<i>B. circulans</i>	≤ 10	255	90	30	115	290	3



SCHEME 1 Structure of EPS and scheme of the disproportionation reaction with maltose as acceptor.

of crystals with maltoheptaose resulted in binding of a maltotetraose molecule in the active site cleft. According to this, the donor (EPS) binds to the enzyme and is cleaved into enzyme-bound G4 and free G3. Then, the acceptor (G_n) binds to the G4-enzyme complex; finally the bound G_n and G4 form a G_{n+4} product which is released from the enzyme. Both EPS that has not reacted and the G_{n+4} product can not be hydrolyzed by the α -glucosidase because both products are blocked at the non-reducing end. Only free G3 is hydrolyzed by the α -glucosidase action liberating *p*-nitrophenol (Scheme 1).

The influence of the chain length of the malto-oligosaccharide acceptor (G1–G7, G1 being glucose) on the disproportionation activity was analyzed. In all cases, the disproportionation activities of CGTase from *Thermoanaerobacter* sp. were notably higher than those using the enzyme from *B. circulans* (Tab. II). The best MOS acceptor for both CGTases was maltose. This result is in agreement with Nakamura *et al.* (1994) who reported that the k_{cat}/K_m value for maltose was 20 times larger than for D-glucose, indicating that the acceptor binding site of CGTase can recognise at least two glucopyranose moieties. 3D Structures of *B. circulans* CGTase always show 2 glucose molecules in the acceptor binding sites (Uitdehaag *et al.*, 1999). Three glucose molecules have been found in the *Thermosulfurigenes* CGTase (Wind *et al.*, 1998).

When increasing the degree of polymerisation of the acceptor, the disproportionation activity decreased, but at G6–G7 this effect was more

TABLE II Disproportionation activities of CGTases at 60°C using different malto-oligosaccharides (G1–G7) as acceptors. Reaction conditions are described in Materials and Methods

CGTase	Disproportionation activity (U/mg protein)						
	G1	G2	G3	G4	G5	G6	G7
<i>Thermoanaerobacter</i> sp.	395	745	725	470	365	325	250
<i>B. circulans</i>	115	290	205	145	110	70	35

pronounced in the case of *B. circulans*. A bulk (steric) factor possibly plays a major role in diminishing the occupation of the binding site in the enzyme. However, the acceptor binding site of CGTase from *Thermoanaerobacter* seems to be more versatile than that of *B. circulans*; even when using maltoheptaose as acceptor a high activity remains.

Acceptor Reaction(disproportionation) with D-glucose: MOS Synthesis

The acceptor reaction with soluble starch (Paselli SA2) as donor and D-glucose as acceptor was studied thoroughly using the CGTase from *Thermoanaerobacter* sp. in view of its excellent disproportionation activity. It was previously reported that D-glucose is one of the best acceptors for CGTase (Norman and Jorgensen, 1992). In fact, the basic requirement for good acceptor performance with CGTase is the presence of a D- or L-glucopyranoside structure with equatorial unsubstituted hydroxyl groups at C-2, C-3 and C-4 (Rendleman, 1996). In the disproportionation/acceptor reaction, a mixture of linear oligosaccharides is formed, most of these may also act as acceptors subsequently. As a result, a homologous series of products with maltodextrin residues attached to the O-4 of the non-reducing end of the acceptor are obtained.

The aim of this work was to manipulate the reaction conditions in such a manner that of the four reactions catalyzed by CGTase, disproportionation was the dominant process. The presence of co-substrate molecules that are strong acceptors (and, consequently, strong promoters of the formation of linear transfer products) clearly will strongly reduce CD production.

As shown in Figure 1, in the presence of a 2-fold weight excess of D-glucose, the formation of CDs from soluble starch is negligible. The optimal temperature of CGTase from *Thermoanaerobacter* sp. for production of CDs is 85°C. The acceptor reaction was studied at 60°C as well. We observed that the yield of the acceptor products was quite similar at 60°C and 85°C (79% vs. 83%, Fig. 2), but the formation of undesirable coloured

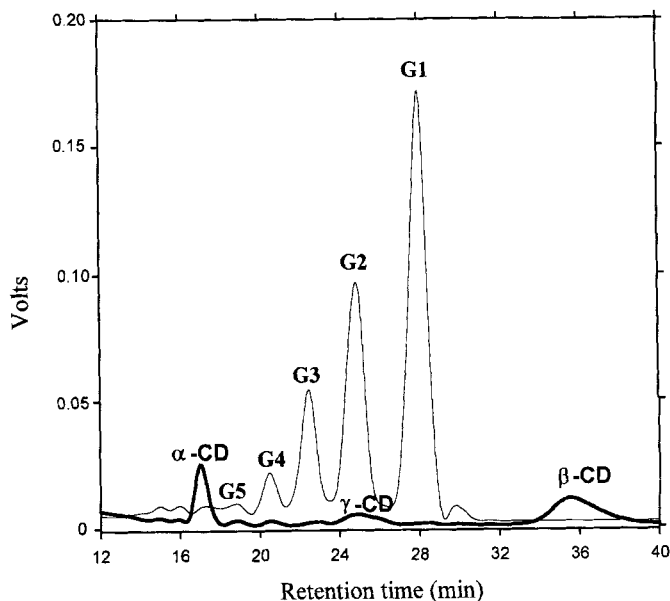


FIGURE 1 HPLC chromatograms of products synthesized in the production assay in presence of D-glucose (thin line) and in absence of acceptor (thick line). Conditions: 0.7 $\mu\text{g/ml}$ *Thermoanaerobacter* CGTase, 10% (w/v) soluble starch, 20% (w/v) D-glucose, 60°C, 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM CaCl_2 , incubation for 48 h.

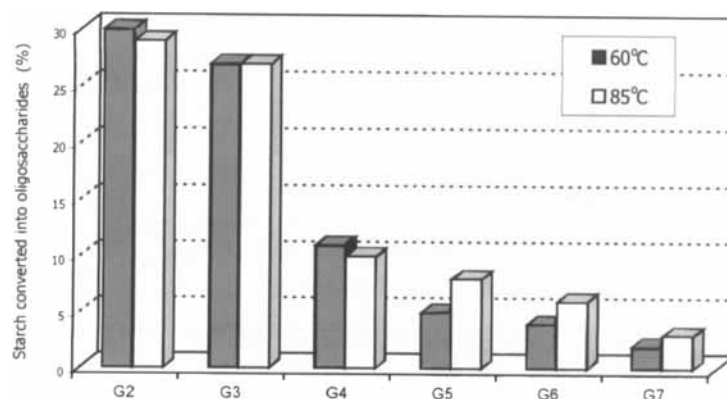


FIGURE 2 Effect of temperature on maltooligosaccharide production in the acceptor reaction with D-glucose catalyzed by *Thermoanaerobacter* CGTase (0.7 $\mu\text{g/ml}$). Conditions: 10% (w/v) soluble starch, 20% (w/v) D-glucose, 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM CaCl_2 . Starch conversion refers to the percentage of initial soluble starch transformed into malto-oligosaccharides at 48 h.

caramelization products (furfurals *via* the Maillard reaction) was much lower at 60°C than 85°C. In consequence, we used this temperature for further studies.

The effect of the ratio (w/w) starch:D-glucose (from 5:1 to 1:2) on product distribution, using 10% (w/v) soluble starch and varying D-glucose

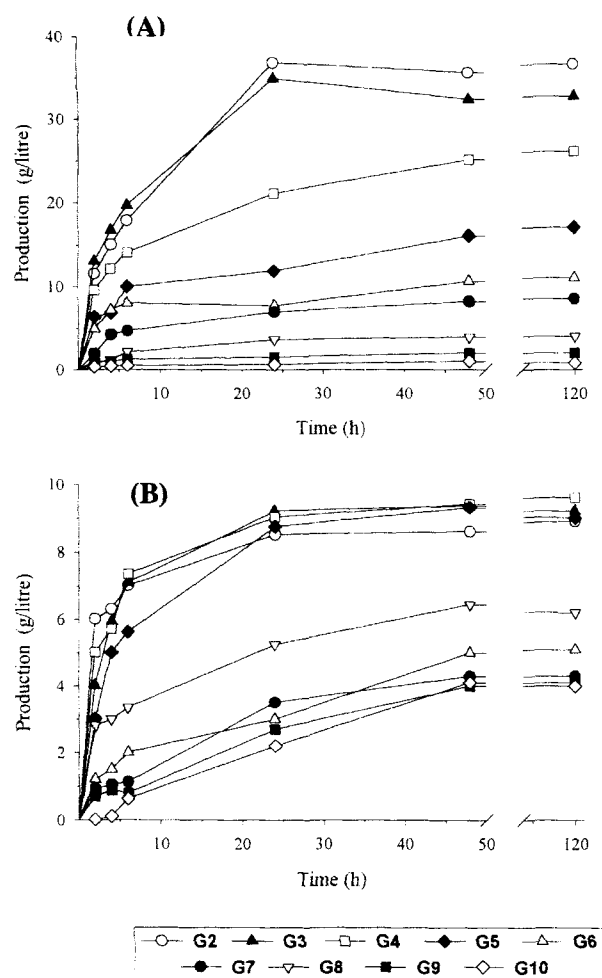


FIGURE 3 Malto-oligosaccharide production in time employing ratios of starch:D-glucose (w/w) 1:1 (A) and 5:1 (B) using *Thermoanaerobacter* CGTase (0.7 µg/ml). Conditions: 10% (w/v) Paselli, with 10% and 2% (w/v) D-glucose for (A) and (B), respectively, 60°C, 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM CaCl₂.

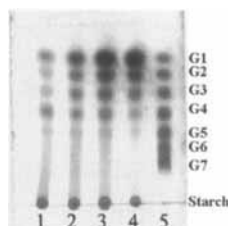


FIGURE 4 TLC analysis of the products in the reaction mixture after 24 h of incubation of *Thermoanaerobacter* CGTase (0.7 $\mu\text{g}/\text{ml}$) with different initial ratios (w/w) starch:D-glucose. Lane 1, ratio 5:1; Lane 2, ratio 2:1; Lane 3, ratio 1:1; Lane 4, ratio 1:2. Lane 5 shows the standards used: glucose (G1) to maltoheptaose (G7). Conditions: 10% (w/v) Paselli, 60°C, 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM CaCl_2 .

concentration, was tested. The equilibrium – regardless of the ratio employed – was practically reached at 48 h (Fig. 3). Figure 4 shows the TLC analysis of the products in the reaction mixtures after 24 h incubation using different initial ratios donor/acceptor. Table III summarizes the conversion of starch into MOS and CDs as a function of this ratio.

In the absence of D-glucose, the consumption of starch was directed preferentially to CDs (34% conversion after 48 h incubation, with a ratio $\alpha:\beta:\gamma$ 2.2:3.6:1). In addition, approx. 4.5% of initial starch was transformed into MOS. When D-glucose is used as acceptor, the synthesis of CDs was notably reduced, and the production of MOS increases significantly. Using a starch/D-glucose ratio 5:1 the yield of MOS increased to 43% and minor amounts of CDs (7%) were formed. As shown in Table III, the lower the percentage of D-glucose, the higher the degree of polymerisation of the acceptor products. Thus, using a ratio donor/acceptor 1:2 a notable yield of MOS (79%) was obtained, and the reaction was quite selective for the formation of G2–G3. The efficiency of starch conversion was at least 2-fold higher for acceptor reaction compared with CD synthesis (79% vs. 34%, Tab. III), probably due to the very notable disproportionation activity of this CGTase.

The selective formation of short-chain malto-oligosaccharides by CGTase may find industrial application. Nakakuki (1993) reported that the consumption of maltotetraose-rich corn syrup reduces the levels of intestinal putrefactive bacteria in humans. In addition, maltotriose is currently used in food products because of its humectant properties, resistance to crystallization, low viscosity, and stability in acid and high temperature conditions (Takasaki *et al.*, 1991).

TABLE III Products of the acceptor reaction catalyzed by the CGTase from *Thermoanaerobacter* sp. using starch as donor and D-glucose as acceptor after 48 h incubation. Reaction conditions are described in Materials and Methods

Initial ratio (w/w) starch: D-glucose	Products formed (g/litre)										Conversion of starch (%) ^a			
	G2	G3	G4	G5	G6	G7	G8	G9	G10	α -CD	β -CD	γ -CD	G2-G10 ^b	CDs
Control ^c	4	1.5	1	1	11	18	5	4.5	34
5:1	8	9	9	7.5	6	6	4.5	3	3	2	4	1	43	7
2:1	20	24	17	11	8.5	5	2	1	1	63	...
1:1	42	27	19	11	7	5	2	1	0.5	76	...
1:2	60	40	15	6	5	2	79	...

^a Percentage of initial soluble starch transformed into malto-oligosaccharides and cyclodextrins after 48 h incubation at 60°C.

^b Conversion of starch into malto-oligosaccharides was calculated subtracting the glucose moiety supplied by the acceptor.

^c Reaction in absence of D-glucose.

CONCLUSION

CGTase from *Thermoanaerobacter* sp. displays the highest disproportionation activity of all CGTases described. With starch as donor substrate, this enzyme is able to use D-glucose as acceptor, with excellent yields of malto-oligosaccharides (> 70%). Our data show that a low donor/acceptor ratio is crucial to strongly reduce cyclodextrin synthesis and to modulate the selectivity of the process. Application of this enzyme will provide significant process advantages with regard to saving costs and reducing undesirable by-product formation. The method described in this work for the synthesis of short-chain malto-oligosaccharides is clearly more simple than the current industrial process where three different enzymes are used. Moreover, many exciting new applications may emerge from employing distinct acceptors in the acceptor reaction catalyzed by the CGTase from *Thermoanaerobacter* sp.

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